A Model for in Vitro Studies of Epidermal Homeostasis: Proliferation and Involucrin Synthesis by Cultured Human Keratinocytes During Recovery After Stripping Off the Suprabasal Layers

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In normal epidermis there is a balance between the rate of cell division and the rate of terminal differentiation. This can be perturbed by injuries such as wounding or tape-stripping, but is reestablished during recovery. The aim of the studies described in this report was to assess whether cultures of human keratinocytes could be used as an experimental model for investigating the mechanism by which epidermal homeostasis is established and maintained. The suprabasal layers were stripped from confluent keratinocyte cultures by incubation in low-calcium medium (0.1 mM calcium ions). After return to normal medium (2 mM calcium ions), the basal layer regenerated a stratified culture of approximately the same thickness as controls. The kinetics of proliferation and terminal differentiation were monitored by measuring the total number of cells and proportion of involucrin-positive cells at intervals before, during, and after stripping. During recovery the proportion of cells expressing involucrin, assessed by immunofluorescence microscopy and polyacrylamide gel electrophoresis, rapidly returned to control levels, but the total number of cells per dish rose more slowly and often failed to reach control values. Thus, terminal differentiation was initially stimulated at the expense of proliferation. Our in vitro model of epidermal regeneration should provide a useful complement to intact skin and animal models for analyzing epidermal homeostasis. *(J Invest Dermatiol 90:739–743, 1988)*

The epidermis is a tissue that is continuously renewed throughout adult life: the rate of production of new cells by division in the basal layer is balanced by the rate of shedding of terminally differentiated cells from the outermost layers [1]. The mechanism by which normal epidermal homeostasis is established and maintained is an important topic for research because uncoupling of proliferation and terminal differentiation occurs in hyperproliferative disorders and malignancy. One way in which to study homeostasis is to challenge the epidermis by injury, for example by tape-stripping, wounding, or exposure to chemicals. In response to injury there is usually an increase in proliferation after a lag period of several hours; then normal homeostasis is gradually reestablished over a period of days [2–4].

Under suitable conditions, human keratinocytes in culture grow as stratified sheets in which proliferation is restricted to the basal layer and terminal differentiation takes place in the suprabasal layers [5]. The aim of the experiments described in this report was to assess whether the cultures can be used as a model to study epidermal homeostasis. If so, they would provide a useful complement to studies on intact skin, as the behavior of populations of keratinocytes could be investigated in the absence of underlying dermis, blood vessels, and nerves. Furthermore, cultures could be used to test potential therapeutic agents.

In the experiments described here we have investigated whether a steady state between proliferation and terminal differentiation is established in culture and have monitored the effect of stripping off all the suprabasal layers on subsequent proliferation and terminal differentiation. The marker of terminal differentiation that we have used is expression of involucrin, the major precursor of the cornified envelope [6]. Whereas in normal epidermis involucrin synthesis begins when cells have migrated one half to two thirds of the distance to the tissue surface [7], the onset of synthesis in culture is immediately suprabasal [8,9].

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Abbreviations:
DME: Dulbecco's modified Eagle's medium
PBS: phosphate-buffered saline
PAGE: polyacrylamide gel electrophoresis

MATERIALS AND METHODS

Cell Culture Human keratinocytes from newborn foreskin (Strain a, fifth to ninth passage) were grown in the presence of a feeder layer of mitomycin C-treated 3T3 cells [10,11]. The culture medium contained 1 part Ham's F12 medium plus 3 parts Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal calf serum, 1.8×10^{-4} M adenine, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 10^{-10} M cholera toxin, and 10 ng/ml epidermal growth factor [12]. EGF, prepared by Dr. George-Nascimento, was generously donated by Chiron Corporation, Emeryville, CA.

Low-calcium medium was prepared by omitting calcium salts from the F12 and DME formulations and depleting the fetal calf serum of divalent cations by treatment with Chelex 100 resin (BioRad Laboratories) [13]. The calcium ion concentration was approximately 2 mM in normal keratinocyte medium and 0.1 mM in low-calcium medium [14].

Selection of Basal Keratinocytes Keratinocytes were harvested with 0.05% trypsin and 0.01% EDTA, then filtered through nylon

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monofilament cloth (11-μm aperature; Tetko), as described previously [14]. Approximately 95% of the cells that passed through the filter were judged to be basal keratinocytes because they did not contain involucrin [15], and these cells were used to set up the experimental cultures.

Experimental cultures were seeded with 10^5 filtered keratinocytes per 60-mm-diameter Petri dish (Falcon). Duplicate dishes were harvested at intervals.

Stripping Off the Suprabasal Cell Layers Confluent stratified cultures in normal medium were rinsed once with 0.02% EDTA and transferred to low-calcium medium. After approximately 3 days the suprabasal layers were detached by aspiration with low-calcium medium. The remaining basal layer of cells was returned to normal medium to allow restratification.

Determination of Cell Number and Percentage Involucrin-Positive Cells At intervals after seeding, duplicate dishes of cells were harvested in 0.05% trypsin and 0.01% EDTA and counted in a hemocytometer. After counting, the cells were resuspended in medium at a concentration of 1.2×10^6 cells/ml. Single drops of the cell suspension were air-dried onto glass coverslips at 37°C for 15 min. The cells were fixed in 3.7% formaldehyde in PBS for 8 to 15 min at room temperature, then permeabilized in absolute methanol for 5 min on ice. This method is based on one described previously [15].

Fixed and permeabilized cells were stained with rabbit antiserum to involucrin followed by fluoresceinated goat anti-rabbit IgG (Miles). The antiserum to involucrin recognized a single band, corresponding to involucrin, in immunoblots of total keratinocyte proteins (results not shown). Incubations with antisera were for 45 min at room temperature followed by thorough rinsing in calcium- and magnesium-free phosphate-buffered saline (PBS).

Stained preparations were mounted in Gelvatol (Monsanto) and examined with a Zeiss photomicroscope III. Photographs of fields selected at random were taken using Kodak Plus X film, developed in Diafine (Acufine Inc.), and printed on Ilford paper. The percentage of involucrin-positive cells was determined from the photographs: at least 200 cells were scored per dish.

Sections Through Keratinocyte Cultures Keratinocyte cultures were detached intact from the culture dish by incubation with 2.5 mg/ml dispase (Boehringer), draped over a filter paper support, fixed in formaldehyde, dehydrated, and embedded in paraffin wax, as described previously [11]. Sections (5 μm) were dewaxed, rehydrated, and stained for immunofluorescence with antiserum to involucrin, as described earlier.

Polyacrylamide Gel Electrophoresis of Cell Extracts Dishes of keratinocytes were harvested as follows. Any remaining 3T3 cells were removed by aspiration with 0.02% EDTA [16]. Keratinocytes were then scraped from the culture dish with a rubber policeman into 10 mM EDTA in PBS and homogenized with a Dounce homogenizer. The extracts were centrifuged for 10 min at 10,000g. The protein concentration of the supernatants was measured using the Bio-Rad automated protein microassay, based on the method of Bradford [17], according to the manufacturer's instructions.

Cell supernatants (25 μg protein) were mixed with sample buffer and run under reducing conditions on a 7.5% gel, using the buffer system of Laemmli [18]. Gels were stained with 0.1% Coomassie Brilliant Blue R (Sigma), destained, and photographed. Polyacrylamide gel electrophoresis (PAGE) of cell pellets confirmed that about 90% of the total involucrin was in the cell supernatants, and that the small proportion in the pellets was approximately constant in different experimental samples.

RESULTS

Culture Morphology and Number of Cell Layers Before and After Stripping The first goal of the experiments was to discover whether the suprabasal layers of confluent, stratified cultures could be removed and, if so, whether the remaining basal layer would regenerate new suprabasal layers. The method tested was to transfer cultures into medium containing a low concentration of calcium ions, as calcium had previously been found to be necessary for the maintenance of intact desmosomal junctions [19].

Figure 1 illustrates the effect of the switch to low-calcium medium. After 2 days in low-calcium medium the suprabasal layers began to detach (compare Fig 1*a* and *b*), and after a further 1 to 2

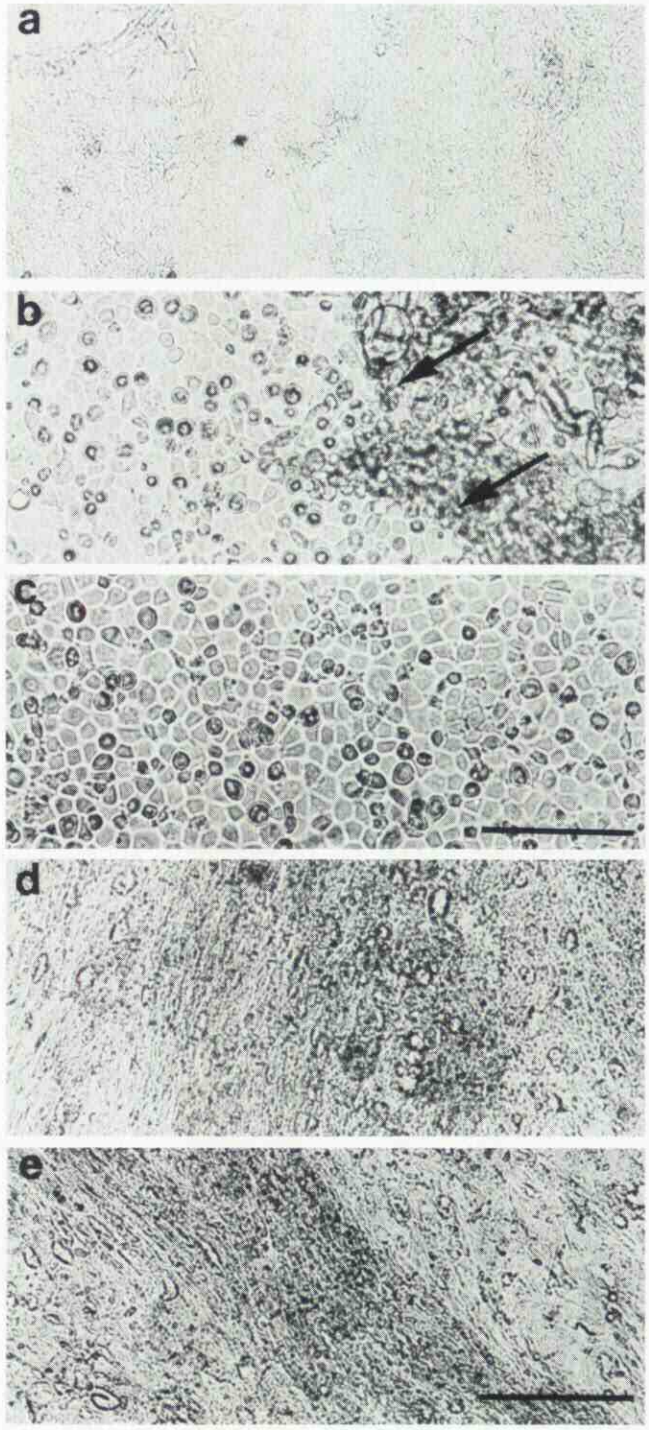

Figure 1. Bright-field photographs of control, stripped, and recovered cultures. *(a)* Confluent dish, 18 days after seeding. *(b)* Confluent dish transferred to low-calcium medium for 4 days. Note basal layer of cells with large intercellular spaces, and clusters of suprabasal cells at the right-hand side of the photograph *(arrows)*. *(c)* Basal layer remaining after removal of suprabasal cells. *(d)* Stripped culture returned to normal calcium medium for 12 days; note morphology similar to that in *(e)*. *(e)* Control dish, 35 days after seeding; note desquamation of uppermost layers. *Bars* = *(b,c)* 200 μm and *(a,d,e)* 500 μm.

days any remaining suprabasal cells could be removed by aspiration (Fig 1*c*). Addition of normal medium (i.e., containing 2 mM calcium ions) to the stripped monolayers for several days led to restratification and acquisition of culture morphology similar to that of controls (Fig 1*d,e*). Shedding of cells from the outermost layers of control cultures occurred after a week or more at confluence, and was also observed in recovered cultures (compare Figs 1*a* and 1*d,e*). Cultures that had recovered once from stripping could be restripped and would recover again; however, some thinning of the basal layer was usually noted.

To assess the number of cell layers in control, stripped, and recovered cultures, and the position of cells expressing involucrin, paraffin wax sections of the cultures were prepared and stained for immunofluorescence microscopy with antiserum to involucrin (Fig 2). Control confluent cultures were approximately 6 to 10 layers thick, with involucrin absent from the basal layer and expressed by all suprabasal cells (Fig 2*a*). After stripping, the suprabasal cells were removed and the remaining basal layer contained predominantly involucrin-negative cells (Fig 2*b*). In cultures that had recovered from stripping (Fig 2*c*) the overall thickness was the same as in control cultures, again with involucrin expressed in all the suprabasal layers.

Proliferation and Terminal Differentiation Kinetics Before and After Stripping Cells that had been filtered to enrich for involucrin-negatives were seeded at 10^5 per 60-mm-diameter Petri dish and duplicate dishes were harvested at intervals. Figure 3 shows the results of a typical experiment in which cell number and the proportion of involucrin-positive cells were measured at intervals up to confluence, after stripping, and during recovery from stripping. Cell number increased logarithmically up to day 15, and remained constant at about 4×10^6 until day 22. It was not possible to follow control cultures for longer periods because with increasing time after confluence, the cells became increasingly difficult to dissociate for counting and staining.

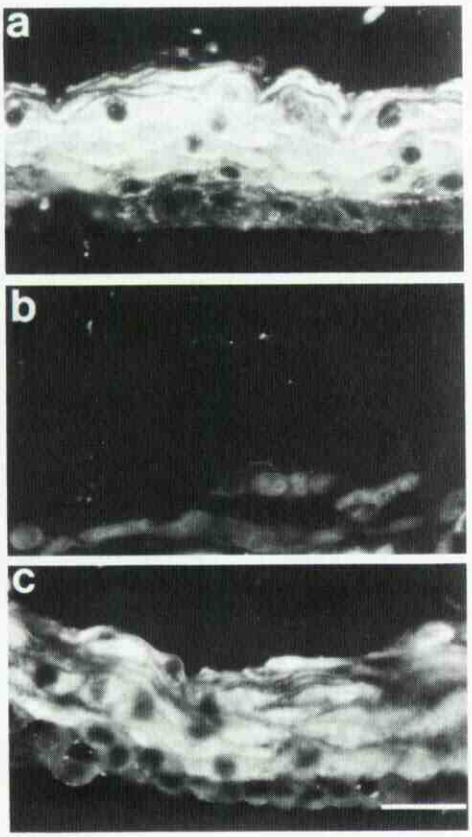

Figure 2. Immunofluorescence photographs of culture sections stained with antiserum to involucrin. *(a)* Confluent dish, 22 days after seeding. *(b)* Stripped culture. *(c)* Stripped culture after 13 days of recovery. *Bar = (a,c)* 50 μm and *(b)* 34 μm.

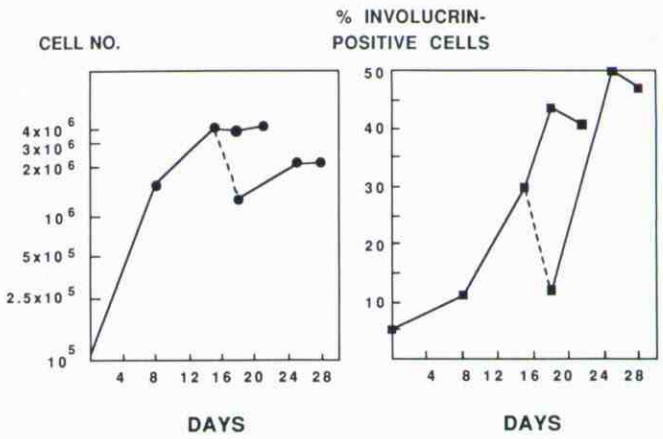

Figure 3. Cell number and proportion of involucrin-positive cells before and after stripping off the suprabasal layers. Each point represents the mean of duplicate dishes which did not vary significantly. The same cultures were used for measuring cell number and percentage involucrin-positive cells.

The proportion of involucrin-positive cells in the starting (filtered) population was 5%. This rose to 11% after 8 days, and more rapidly thereafter, reaching a maximum of 43% on day 18. On the final day, day 22, the proportion of involucrin-positive cells was 41%. Thus, the proportion of cells expressing involucrin continued to rise for 3 days after cell number had leveled off, and was approximately constant for 4 days thereafter.

Stripping off the suprabasal layers led to a drop both in total cell number and in the proportion of involucrin-positive cells. The number of involucrin-positive cells never fell below about 10% (Fig 3). This is probably because stripping took at least 3 days, and during that time, when stratification was inhibited, involucrin-positive cells began to accumulate in the layer attached to the tissue culture plastic [14].

During recovery from stripping there was a gradual increase in the number of cells per dish, often after an initial lag of 2 to 3 days. In the experiment shown in Fig 3, cell number leveled off about 7 days after stripping, but at a lower value than the confluent density of the unstripped cultures. Control values were sometimes reached in other experiments, but this took up to 3 weeks after stripping. In cultures stripped for a second time cell number never reached control values (results not shown). In contrast to the changes in cell number during recovery, the proportion of involucrin-positive cells rose steeply and returned to, or exceeded, control levels within a week (Fig 3). When cultures were stripped for a second time, the proportion of involucrin-positive cells again rose rapidly to control levels or higher.

These experiments suggested that during recovery from stripping the cultures might initially be biased toward terminal differentiation at the expense of proliferation. To investigate this, the proportion of involucrin-positive cells was plotted versus cell number in control cultures and cultures recovering after one or two strippings (Fig 4). In control cultures (■), as expected from Fig 3, the proportion of involucrin-positive cells was higher at confluent cell densities ($\geq 3 \times 10^6$) than at lower densities ($5 \times 10^5 - 2 \times 10^6$), although there was considerable scatter at high cell density.

In cultures recovering from one stripping (□) the maximum proportion of involucrin-positive cells (35–55%) was reached at lower seeding densities than in control cultures (i.e., the points were shifted toward the vertical axis; Fig 4). Parallel lines could be fitted to the control and once-stripped points; the vertical distance between the two lines was 12.937 ± 2.678 (mean ± standard error), indicating a significant difference between them. Too few data points were available to allow similar analysis of cultures that had been stripped twice (△), but the trend appeared to be a further

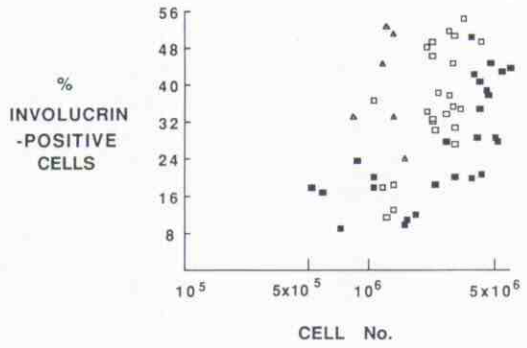

Figure 4. Graph of percentage involucrin-positive cells versus total cell number in control cultures (■) and in cultures recovering after stripping for the first (□) or second (△) time. These results represent pooled data from seven different experiments.

reduction in the cell density at which the maximum percentage of involucrin-positives was reached (Fig 4).

Involucrin Content of Stripped and Unstripped Cultures
The amount of involucrin in the cultures was assessed by PAGE of soluble cell extracts (Fig 5). In stripped cultures *(track 3)* the involucrin band was very faint; after 4 days of recovery a stronger band was visible *(track 4)* and the band approached control levels by 18 days of recovery (compare *tracks 2 and 5*). Thus, the amount of involucrin in the cultures correlated well with the changes in the proportion of cells expressing involucrin.

DISCUSSION

The aim of the experiments described in this report was to investigate whether cultures of human epidermal keratinocytes could provide a model for studying epidermal homeostasis and its reestablishment after injury. We found that all the suprabasal layers could be stripped off by transferring confluent, stratified cultures to low-calcium medium, as calcium ions are required for the maintenance of desmosomal junctions [19]. When stripped basal layers were returned to normal medium, the cells restratified and cultures of normal thickness and morphology were regenerated. The cultures could be stripped for a second time, although recovery was slower.

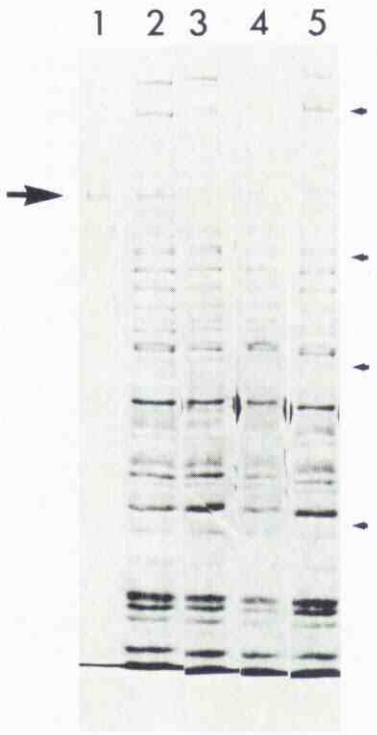

Figure 5. A 7.5% polyacrylamide gel stained with Coomassie Blue. *Arrowheads* indicate positions of molecular weight markers (D): 200,000; 97,000; 68,000; and 43,000. *(1)* Purified involucrin marker *(large arrow)*. *(2)* Control culture, 18 days after seeding. *(3)* Stripped culture. *(4)* Stripped culture, 4 days recovery. *(5)* Stripped culture, 18 days recovery.

The kinetics of proliferation and terminal differentiation in the cultures were monitored before and after stripping by measuring cell number and the percentage of cells expressing involucrin. Approximately 4 days after confluence the number of involucrin-positive cells stopped rising and appeared to level off. By the criteria of constant cell number and proportion of terminally differentiating cells, homeostasis was achieved; however, we could not determine how long the balance was maintained because from about 5 days after confluence, the cultures were impossible to disaggregate for counting. Cultures that were maintained for 7 days or longer after confluence did begin to shed cells from the uppermost layers into the medium (compare Figs 1*a,e*) and occasional thinning of older cultures (14–21 days postconfluence) suggested that there was a net loss of cells (Read and Watt, unpublished observations). Thus, homeostasis was probably not maintained throughout the entire life span of culture.

After stripping, the total number of cells and proportion of involucrin-positive cells dropped. The proportion of involucrin-positive cells returned to control levels, or slightly higher, within 4 days, but the increase in total cell number was slower and often leveled off at a lower value than in control cultures. In cultures recovering from stripping, the maximum proportion of involucrin-positive cells (35–55%) was attained at relatively lower cell densities than in controls, a trend that was more marked in cultures stripped twice than cultures stripped once. PAGE analysis of the amount of involucrin in control, stripped and recovered cultures confirmed the immunofluorescence data.

Although the number of cells in recovered cultures was usually slightly lower than in the controls, the overall thickness of control and recovered cultures was the same and involucrin-positive cells were rarely seen in the basal layer. This suggests that there may have been cell enlargement in the basal layer during recovery, as noted in vivo [2]. By the criteria of culture thickness and proportion of cells expressing involucrin, the cultures were able to reestablish homeostasis after injury.

In interpreting the response of the cultures to stripping, it is important to consider the significance of both the proportion of involucrin-positive cells and the absolute number of involucrin-positive cells. Thus, in the experiment shown in Fig 3, although the stripped cultures at day 25 contained a higher proportion of involucrin-positive cells than the day 15 controls, the absolute numbers of involucrin-positive cells per dish were similar, because there were more cells in the control than the stripped cultures. It seems that the primary response to stripping was an increase in the proportion of terminally differentiating cells, but this could be due to either an increased terminal differentiation rate or a reduced rate of proliferation. As withdrawal from the cell cycle and induction of terminal differentiation are normally tightly coupled, further experiments will be required to distinguish between the two possibilities.

Although cultures of keratinocytes resemble epidermis in situ in regenerating the suprabasal layers after injury there is an important difference. In vivo, after a short lag, there is a wave of mitosis, resulting in transient hyperproliferation; then homeostasis is reestablished within a few days [2–4]. In vitro homeostasis was also reestablished, but increased terminal differentiation appeared to be the first response to stripping, with an increase in cell number occurring several days later and often failing to attain control levels. Stripping did not reduce the plating efficiency of the cells relative to control, unstripped, cultures (Watt, unpublished observation), suggesting that the lack of hyperproliferation was not due to cell injury.

One reason for the difference between the in vivo and in vitro responses may be that there is no analogous treatment in vivo to stripping off all the suprabasal layers while leaving the basal layer intact. Perhaps more important, though, is the evidence that cul-

tured keratinocytes are already hyperproliferative, by a number of different criteria, including shortened cell cycle time [20] and premature involucrin expression [9,21], and so may be unable to divide any faster or expand their growth fraction in response to injury.

In conclusion, keratinocyte cultures have the capacity to regenerate their suprabasal layers after stripping, and during the recovery process there is an initial bias toward terminal differentiation at the expense of proliferation. This experimental model provides a useful new technique for investigating how proliferation and terminal differentiation are coupled, complementing intact skin and animal models. Future experiments of interest will be to investigate other markers of terminal differentiation; to test the effect of dermis [9] on the recovery process; and to discover whether the kinetics of recovery are altered when keratinocytes are forced to grow more slowly by omitting growth factors from the medium. Finally, the effect of potential therapeutic agents, such as retinoids, on recovery will be of interest.

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